

THE CUPRIC COMPLEXES OF GLYCINE AND OF ALANINE

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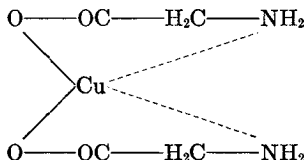
I. INTRODUCTION

(a) HISTORICAL

The following report is the first of a projected series of studies of the physical chemistry of the compounds of the heavy metals, particularly of copper and of iron, with substances of biological importance. These studies are invited by the accumulation in recent years of examples of the importance of the heavy metals in biological chemistry.

The copper compounds of glycine and of alanine were studied first, in the hope that the analysis of the factors affecting the formation of these relatively simple compounds would facilitate the elucidation of the more complex systems.

It has long been known that amino acids and cupric ion react to form stable complexes. Ley, in 1904 (1), suggested for the copper-glycine complex the formula



the dotted lines signifying secondary valencies. In support of this formula Ley and his collaborators later published spectrophotometric absorption data in the visible and ultra-violet regions for copper-glycine and for copper-alanine (2, 3). The chief evidence adduced by Ley for the accompanying formula was the similarity between the absorption spectra, in the visible region

of the copper complexes of the monoaminomonocarboxylic acids and those of the cupriammonium complexes.

Barker, in 1907 (4), reported the results of potentiometric and freezing point measurements in aqueous solutions of copper sulfate and glycine, from which the conclusion was drawn that 4 molecules of glycine were combined with 1 of copper, the combination being effected by the four secondary valencies of the copper with the nitrogen atoms of the amino acids. Most of the results published by Barker, without special interpretation, may be taken equally well to indicate that 3 instead of 4 molecules of glycine are combined with 1 of copper.

The interpretation of freezing point data here is complicated by the formation of aggregates between the amino acid molecules (5), which has only been discovered in the last few years, and which the earlier investigators failed to take into account. An additional uncertain correction must be introduced also for the activity coefficients of the electrolytes in solution.

These qualifications also weaken the evidence adduced by Pfeiffer (6) from freezing point measurements for the formation of complexes between neutral salts and amino acids. Direct evidence that these compounds may exist at least in the solid state was, however, obtained by Anslow and King (7), who crystallized a number of complexes of inorganic neutral salts and dicarboxylic amino acids.

In a series of interesting and suggestive studies Kober and Sugiura (8) and Kober and Haw (9) showed that cupric ion forms with monocarboxylic- α -amino acids compounds of the type CuA_2 , where A refers to the amino acid molecule; and with glutamic acid, aspartic acid, isoserine, and all polypeptides, compounds of the type CuA . From the examination of a large number of compounds they deduced the rule that the region of absorption is shifted more and more toward the blue as the number of nitrogen atoms attached to the copper in a stable ring, either by secondary or primary valencies, increases from 2 to 4. Accordingly, in strong alkali monoamino acids and dipeptides give a blue color similar to that of cupriammonia, and an absorption maximum at 6300 Å.; tripeptides, a bluish violet with absorption maximum at about 5400 Å.; and tetrapeptides, a red color with absorption maximum at 4430 Å. The latter is the true biuret color, while the color given by proteins resembles that of the tripeptides.

Pfeiffer (6) has accepted the type of formula proposed by Ley for the copper compounds of glycine and alanine; *i.e.*, an internal complex with the primary valencies of the copper attached to the carboxyl groups. Ley and Pfeiffer, in their formulation of the copper compounds of the amino acids, have tacitly accepted the classical conception of the strength of the acid and basic radicals of the amino acids. Since the *Zwitter Ion* constitution of amino acids seems now firmly established (10), we have employed it throughout in designating the species of ionic form of the amino acid existing at any given hydrogen ion concentration.

The interesting point which the present study has revealed is the variation of the constitution of the copper-glycine or copper-alanine complexes present in any solution with the hydrogen ion concentration. This phenomenon seems, up to now, to have been almost entirely overlooked. Ley and Hegge (2) state that copper-glycine can be obtained in two forms, in plate-like and needle-like crystals; but that no difference is detectable in solution. By conductivity and electrode potential measurements Barker found that the addition of glycine to solutions of zinc sulfate increased the hydrogen ion concentration of the solution; and from the similarity in behavior of the conductivities of glycine-zinc and glycine-copper sulfate solutions he concluded that the formation of a glycine-copper sulfate compound also increased the hydrogen ion concentration of the solution. This we have confirmed. Kober and Haw observed that the absorption of a given complex is somewhat dependent on the concentration of the hydroxyl ions. As far as the amino acid complexes are concerned they reported only that, "In the amino-acid blue complexes there is no visible change with weak alkali; but in strong alkali most of the copper is precipitated."

This oversight was due, it seems, to the practice of attempting to account for all the properties of solutions of copper compounds of glycine and of alanine by those of the crystals. This amounts in effect to considering only saturated solutions in which the copper and amino acid are in equivalent proportions. It is obvious that such compounds can be of only remote significance under physiological conditions where the concentration of nitrogenous substances is enormously in excess of that of the copper, which is present only in traces.

One of the few attempts to consider the equilibria in solution was made by Shibata and Matsuno (11). They noted that changes occurred in the absorption spectra of copper sulfate solutions with changes in concentration, but limited their explanations to the postulate of the formation of aquo complexes.

(b) *Absorption Spectra*

The data presented below indicate that as the hydrogen ion concentration is varied from pH values of 0 to 13, at least four compounds of cupriglycine and cuprialanine are formed. In Figs. 1 and 3 the absorptions in the visible spectra of these four compounds are set out and compared with those of copper sulfate, of undissociated copper acetate (*i.e.* in alcoholic solution), and of cupriammonium sulfate.

The effect of varying the hydrogen ion concentration at constant relative concentration of amino acid to copper is shown in Figs. 2 and 4, which give only a few curves from the series of thirty-four obtained. As discussed in Section III, *a* it was impossible to interpret the changes in absorption in terms of less than four compounds. Furthermore, analysis of combined spectrophotometric and potentiometric data showed that in some solutions three copper-amino acid compounds were present at once. On this account it would be extremely difficult, if not impossible, to obtain from aqueous solutions the second acid copper-glycine or alanine complex in the pure crystalline state. Even if these compounds were prepared, on re-solution a rearrangement would occur, with the result that the solutions so formed would contain more than one complex. The same consideration, in lesser degree, applies to the other complexes. The pure neutral compound, if prepared and dissolved in water, would probably suffer the least change, though even here the absorption data of Ley and of Kober and Haw suggest that a detectable amount of the second acid complex is formed (*cf.* p. 683).

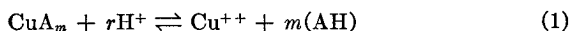
Since a solution of the crystals of any one copper-glycine or alanine complex would therefore at once become a mixture, the only way these compounds can be obtained alone in solution is by increasing the concentration of amino acid (with the pH kept constant within a suitable range) until no further change in the absorption of the solution occurs. By this method the absorption

curves of the pure neutral and basic complexes were obtained. The determination of the absorption curves of the two acid complexes—one, designated the first acid complex, predominating at pH 2.0, the other, the second acid complex, predominating at pH about 4.0—presented more difficulty, since their ranges of stability overlap. The curves deduced are therefore less certain than those of the neutral and basic complexes. The curves for the first acid complexes were obtained from dilute solutions of amino acid after the absorption due to the free cupric ion, whose concentration was determined potentiometrically, had been subtracted from the total absorption observed. The curves for the second acid complexes were obtained by a method of trial and error described below.

(c) *Potential Measurements*

By means of copper electrode potential measurements we have also attempted to determine by the following method, due to Bodländer and Storbeck (12), the number of molecules of amino acid combined with 1 molecule of copper.

The general equation for the formation of any one of the complexes (where AH represents that form of the amino acid taking part in the reaction, and CuA_m the complex) is



In it the CuA_m bears a charge which varies with the complex under consideration and also contains $(m - r)$ H atoms. The general treatment of the equilibria is facilitated (without being invalidated in any way) by omitting these from mass law equations. The mass law expression for the equilibrium is therefore

$$\frac{(\text{Cu}^{++}) \cdot (\text{AH})^m}{(\text{CuA}_m) \cdot (\text{H}^+)^r} = K \quad (2)$$

where m is the number of molecules of amino acid in the complex and r the number of hydrogen ions set free in the formation of 1 molecule of complex.

Therefore, for two different amino acid and hydrogen ion concentrations we may write

$$\frac{(\text{Cu}^{++})_1 \cdot (\text{AH})_1^m}{(\text{CuA}_m)_1 \cdot (\text{H}^+)_1^r} = \frac{(\text{Cu}^{++})_2 \cdot (\text{AH})_2^m}{(\text{CuA}_m)_2 \cdot (\text{H}^+)_2^r} \quad (3)$$

which on conversion to logarithms and rearrangement becomes

$$m \log \frac{(\text{AH})_1}{(\text{AH})_2} = \log \frac{(\text{Cu}^{++})_2}{(\text{Cu}^{++})_1} + \log \frac{(\text{CuA}_m)_1}{(\text{CuA}_m)_2} + r(\text{pH}_2 - \text{pH}_1) \quad (4)$$

Since the potential difference between copper electrodes, in two solutions where all other ions except the cupric ions are at practically the same concentration, is

$$E = \frac{RT}{nF} \ln \frac{(\text{Cu}^{++})_1}{(\text{Cu}^{++})_2} \quad (5)$$

therefore, by being converted to base 10 and inserted in Equation 4 the latter becomes

$$m \log \frac{(\text{AH})_1}{(\text{AH})_2} = (E_1 - E_2) \frac{nF}{2.303 RT} + \log \frac{(\text{CuA}_m)_1}{(\text{CuA}_m)_2} + r(\text{pH}_2 - \text{pH}_1) \quad (6)$$

If practically the whole of the copper is in the complex form, the second term on the right-hand side disappears, and hence the value of m , *i.e.* the number of molecules of amino acid combined with 1 cupric ion in a given complex, can be obtained from the potential difference between copper electrodes, if the hydrogen ion concentrations are the same in the two solutions.

In those solutions in which an appreciable fraction of the copper is not in the complex form, a correction for this has to be applied. This was done either by assuming the copper electrode potentials to give absolute Cu^{++} concentrations, and deducting these from the total copper present, or else by algebraic analysis of the absorption curves, as described in Section III, *a*. Where possible, both methods were used, and the results checked each other fairly satisfactorily.

In acid solutions it was found that hydrogen ion concentrations great enough to suppress ionization of the COOH group of the amino acid also suppressed all complex formation (*cf.* Fig. 2, *A*). This means that only the *Zwitter Ion* form of glycine and alanine takes part in the complex formation with copper in the acid range. Consequently, the concentration of free amino acid employed in Equation 6 refers only to that in the *Zwitter Ion* form. This is therefore the difference between the total calculated concentration of *Zwitter Ion* form of amino acid at the pH of the solution and

that bound in the complex. Where the concentration of amino acid was greatly in excess of that of the copper, and the pH between 4.5 and 8.0, no error is incurred by taking as the concentration of free amino acid the total amount added initially.

For the determination of hydrogen ion concentrations in the presence of copper the glass electrode was used, as described in Section II, *b*.

When the value of m had been obtained from solutions in which the pH was the same, *i.e.* where only the amino acid concentrations were different, this value of m was then employed in other solutions for the determination of r . By using several determinations mean values for m and r were obtained, and from these the approximate equilibrium constants were computed.

In the case of the neutral compounds of glycine and of alanine, and of the second acid compound of alanine, another, independent, method of obtaining the value of r was also employed. This consisted in the measurement of the small change in hydrogen ion concentration of the amino acid solution, resulting from the addition of measured small amounts of copper sulfate solution. The method is described in Section III, *b*; the results are shown in Tables III, VII, and IX. In this method, when the amino acid concentration was greatly in excess of that of the copper, all of the copper could be considered to be in complex form; in more dilute solutions, the free copper, and hence by subtraction the amount of complex, was determined by means of the copper electrode.

II. EXPERIMENTAL

(a) *Absorption Spectra*

The absorption data were obtained with a König-Martens spectrophotometer. The cell employed was a T-piece 73.5 mm. long, with plate glass ends cemented on. This length of absorption solution permitted the use of low concentrations of copper (usually 0.002 M) and of correspondingly large variations in the relative excess of amino acid. In order to maintain the composition as uniform as possible with respect to SO_4 ions, which facilitated the interpretation of the copper electrode potentials, K_2SO_4 was added to all solutions to a final concentration of 0.1 M. Owing to the large error which traces of opalescence introduce,

all solutions were filtered into the absorption cell before measurements were made. To allow for absorption due to water and to reflection by the cell surfaces, blank determinations were made on distilled water similarly filtered into the cell.

(b) Hydrogen Ion Concentrations

In the solutions marked * in Tables I, II, and V, a quantity of H_2SO_4 estimated to be equivalent to the hydrogen ions set free by the formation of the copper-amino acid complex was added to the solution of amino acid, together with potassium sulfate, and the acid or alkali necessary to bring the hydrogen ion concentration of the solution to the desired pH. The pH was then measured electrometrically with a Moloney hydrogen electrode (13). The value obtained was checked colorimetrically on the similar solution containing CuSO_4 (instead of the excess acid) on which the spectrophotometric and copper electrode potential measurements were carried out. The pH values given for these solutions are uncertain to ± 0.2 pH units.

In the solutions marked † in Tables I, II, V, VI, and VIII, the hydrogen ion concentrations were measured in the final mixture containing copper. This was done with the glass electrode, the modified electrical arrangement described by Robertson (14) being used. With this set-up, potentials were determined by means of a high sensitivity galvanometer (Leeds and Northrup type 2500) and a type K Leeds and Northrup potentiometer, without the use of vacuum tubes. The electrodes were made from the special Corning glass employed by MacInnes and Dole (15). The electrode used was calibrated before each measurement with buffer solutions whose pH values bracketed that of the solution to be measured. The uncertainty of the values obtained was not greater than 0.02 pH unit.

(c) Copper Ion Concentrations

Copper foil electrodes have been used in complex ion work by Riley (16). The use of foil or wire electrodes, however, was shown by Getman (17) to introduce variations in the potential according to the metallurgical treatment of the copper, and the electrodes here employed were therefore prepared according to the directions of Lewis and Lacey (18) and of Getman (17).

Spongy copper was obtained by electrolyzing a solution of twice recrystallized CuSO_4 between platinum foil electrodes with a high current density, so that the copper was deposited in streamers of spongy metal which did not adhere to the cathode but sank to the bottom of the beaker. This was thoroughly washed in freshly boiled distilled water (redistilled through a block tin condenser) until quite free from SO_4 and then set away in a bottle filled with freshly boiled redistilled water to preserve it from oxidation in the air. The copper electrodes consisted of platinum wire very thinly covered by a film of copper deposited out of a 0.01 M CuSO_4 solution by means of the current from one dry cell for 30 seconds. These electrodes were then covered by spongy copper which had been washed several times with the solution in which the electrode was finally brought to equilibrium. Before being used on amino acid solutions the electrodes were checked by measuring the potential difference between known concentrations of copper sulfate in the two electrodes, usually 0.01 M against 0.001 M. The potential difference found at 25° for this concentration difference was 22 millivolts, the identical value obtained by Labendzinski (19) for this cell. When both CuSO_4 solutions contained 0.1 M K_2SO_4 , values of 29.5 to 30.1 millivolts were obtained. In the operation of this cell it was found that difference in hydrogen ion concentration produces a large liquid junction potential. Accordingly, when the cupric ion potential difference between solutions containing different concentrations of amino acid was desired, the pH values of the standard and the unknown solutions were made as nearly as possible the same. In spite of these precautions discrepancies sometimes occurred between the values for the concentration of free cupric ions, deduced from copper electrode potential, and those obtained from spectrophotometric data. It is possible that this was due to a solution of metallic ion by the acid in the presence of small amounts of dissolved oxygen. In most cases, however, the values found by potentiometric and spectrophotometric methods were in fairly good agreement.

The usual procedure in the determination of the cupric ion potential was as follows: The two electrode vessels, one containing the standard copper sulfate solution, the other the amino acid and consequently an unknown concentration of cupric ions, connected by an intermediate solution which was the same as the

standard, were set away in an air bath at 25°, with the stop-cocks closed. These were opened only while readings were being taken. Fresh liquid junctions were made for each reading by opening screw clamps on top of each electrode vessel. Readings were taken from time to time until the potential was observed not to drift more than 1 millivolt in 1 hour. The time elapsed was as a rule from 2 to 3 hours.

The employment of 0.1 M K_2SO_4 in the standard and in the solutions containing amino acid eliminated potentials due to SO_4 ion concentration difference, and maintained a nearly constant ionic strength in all solutions. It was hoped that this nearly constant concentration of strong electrolyte would minimize the effect of varying amino acid concentrations on the activity of the cupric ion, and, on the assumption of constant ionic strength, would justify the calculation directly from the potentials of cupric ion concentrations instead of cupric ion activities.

The copper sulfate used in the preparation of the spongy copper and in the solutions was twice recrystallized from a c.p. specimen, care being taken to obtain small crystals; and then powdered and dried at 120°. From this salt a stock 0.1 M solution was made which served for all subsequent dilutions.

The specimens of glycine and *dl*-alanine used were recrystallized several times from isoelectric solutions of commercial preparations. After drying, these gave the melting points cited in the literature, 235° for glycine and 295° for alanine.

III. Cupric Salts of Glycine

(a) Absorption Spectra

Fig. 2 shows the variation in the absorption of solutions containing a constant concentration of $CuSO_4$ (0.002 M) and of glycine (0.5 M) when the hydrogen ion concentration is changed from pH 0 to 12. Beginning with the absorption of free $CuSO_4$ in extreme acidity, the absorption in the red rises, at first slowly, then more rapidly, and then falls again. The absorption in the neighborhood of 6250 Å. increases steadily and attains a constant value. From pH 5 to 8 the absorption remains constant. It was concluded, therefore—a conclusion corroborated by all subsequent analysis of the data—that all but a negligible quantity of the copper was bound in the form of only one complex, whose absorption curve was

that found in these solutions, from pH 5 to 8. This compound was designated as neutral copper-glycine.

In alkalinities higher than pH 8 a further increase in absorption was observed in the orange and red end of the spectrum. This was taken to indicate the formation of another compound, which was designated as basic copper-glycine. The extrapolation method by which the absorption curve was established is described below.

All attempts to interpret the absorption curves of the solutions more acid than pH 5 in terms of a neutral complex and of only one acid complex failed. The increasing and then diminishing absorption at the red end of the spectrum could be accounted for completely only by postulating a second acid complex occurring between the first acid and the neutral complexes. Similar results were obtained with alanine.

In Fig. 1 are set out the final absorption spectra of the four salts. The curve for the first acid copper-glycine (Curve 3) was derived from dilute acid solutions (pH 4.4 to 4.6) in which the free cupric ion was calculated from copper electrode potentials, amounting to 63 and 53 per cent of the total copper respectively; the absorption due to this amount of Cu^{++} was deducted from the total absorption. The accuracy of the curve so obtained was confirmed by the absorption of a solution containing 1 M glycine and 0.002 M copper at pH 2.05, where the large excess of glycine caused practically all of the copper to be combined in this form. The absorption of the first acid complex having been thus obtained, it was possible by means of simultaneous equations to solve, with good agreement, the absorption curves of other solutions in the acid range.

The general method for solving such curves is as follows:

Let x = fraction of total copper as cupric ion
 $1 - x$ = " " " " in complex
 A_1 = absorption of cupric ion at wave-length λ_1
 B_1 = " " copper in complex form at wave-length λ_1
 Δ_1 = total absorption of solution at wave-length λ_1
 Then $x A_1 + (1 - x) B_1 = \Delta_1$. Similarly at another wave-length,
 $x A_2 + (1 - x) B_2 = \Delta_2$, etc.

Proceeding through the visible spectrum we obtain a value for x , from the solutions of pairs of simultaneous equations, which will

be constant throughout the spectrum if the absorption curves chosen, *i.e.* the values of $A_1, A_2, A_3, B_1, B_2, B_3$, etc., are correct. Values for A_1, A_2, A_3 , etc., were obtained from a pure copper sulfate solution at high dilution (see Curve 1, Fig. 1). If a constant value of x is obtained for any given set of values for B_1, B_2, B_3 , etc., the accuracy of these values for the absorption spectrum of the unknown compound is established.

The absorption spectrum for the first acid compound so obtained was then combined with that for CuSO_4 to yield concordant

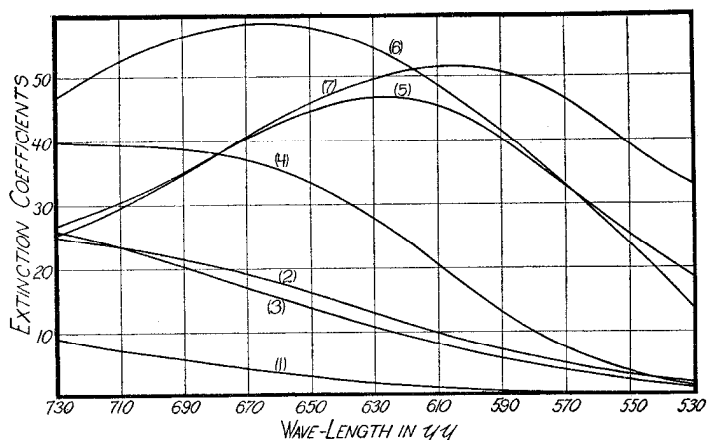


FIG. 1. Absorption curves of cupriglycine compounds. Extinction coefficients $\log \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{L(\text{cm.})}$ at various wave-lengths in $\mu\mu$. Curve 1, copper sulfate; Curve 2, copper acetate in alcohol; Curve 3, first acid cupriglycine; Curve 4, second acid cupriglycine; Curve 5, neutral cupriglycine; Curve 6, basic cupriglycine; Curve 7, cupriammonium sulfate.

figures for the concentrations of each in solutions in which the glycine concentration varied from 1 M to 0.004 M, and the pH from 0.25 to 4.6. In spite of this concordance, it is still possible that this curve is too high. The absorption is so near to that of the CuSO_4 that spectrophotometric data alone cannot lead to a very reliable curve for the first acid complex. If a lower absorption curve were taken, the simultaneous equations would yield different but, within the limits of experimental error, almost concordant values for the amounts of free and bound copper. Further, the curve

given is much higher than that for the corresponding copper-alanine, which is more firmly established. However, there is a good reason for this (see Section IV, *b*), and also the curve is supported by the agreement between potentiometric data for the amounts of free copper and those calculated from the absorption spectra. Its close resemblance to the curve of undissociated cupric acetate is also probably significant.

The absorption spectrum of the second acid compound was constructed arbitrarily, by trial and error, to account for the absorptions of the solutions between pH 3 and 6. This curve (Curve 4 in Fig. 1) was not obtained unmixed with those of other complexes in any solution. The absolute values are accordingly somewhat uncertain. However, combination of this curve with the others satisfactorily accounted for the absorptions of solutions ranging from 0.5 M glycine at pH 2.9 to 0.002 M glycine at pH 7.2. Thus the complex appears to exist in concentrated solutions, *i.e.* when glycine is greatly in excess of copper, around pH 4, and in neutral solutions when the concentrations of glycine and copper are nearly the same and both very dilute.

The neutral copper-glycine is the only form described in the literature. Its absorption curve (Curve 5 in Fig. 1) is based on the absorptions of solutions at pH 5 to 8 in which the concentrations of glycine varied from 0.05 to 1.0 M. Since practically all the copper in these solutions was bound in this form, the absorptions obtained were almost the same in spite of the 20-fold variation in concentration of glycine.

This absorption curve is higher than any previously quoted for this complex. Thus at 6250 Å., where we have found the extinction coefficient to be 47, Kober and Haw give 40, and Ley and Vanheiden (3) give 46. The explanation of these lower values lies in the fact that their solutions were prepared by dissolving crystals of copper-glycine in water, with a resulting total molal concentration of glycine only twice that of the copper. Some decomposition, therefore, occurred into the second acid form, and to a slighter extent into free Cu^{++} and glycine. The absorption would accordingly be less than that of a solution containing only neutral complex. However, the form of the curve given in both instances is the same as that given here.

The existence of a basic copper-glycine complex was indicated

from the change in absorption, clearly visible in the color of the solutions, beyond pH 8. The absorption of this basic form was obtained by extrapolating the values obtained in solutions of increasing alkalinity, containing 0.5 M glycine and 0.002 M copper sulfate. The quantities plotted were the excess absorption over that of the pure neutral complex, for each wave-length measured,

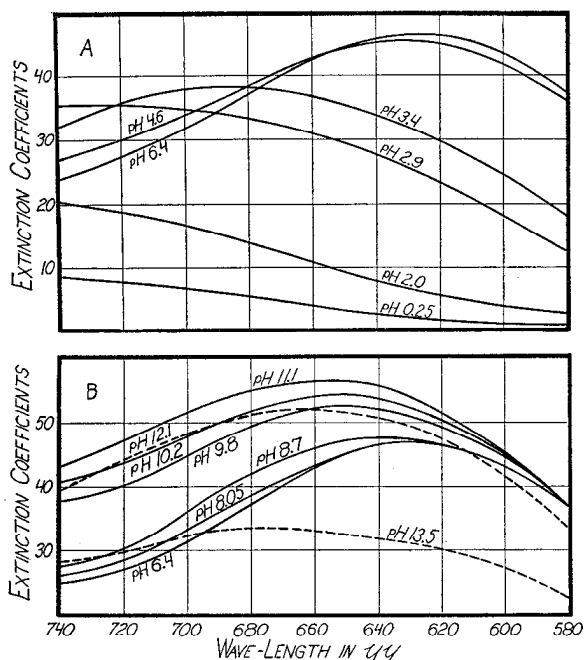


FIG. 2, A. Effect of changing H ion concentration on the absorption of cupriglycine solutions containing the same amounts of copper and of glycine; Cu = 0.002 M, glycine = 0.50 M; acid range.

FIG. 2, B. Same as Fig. 2, A; alkaline range. Solutions in which precipitation occurred are shown in broken lines (pH 12.1 and 13.5).

against pH. The curves so obtained were of the form of dissociation curves, and flattened rapidly towards pH 11.1. From each the absorption at one wave-length was taken, and the resulting extrapolated curve for the basic complex is Curve 6 in Fig. 1. By combining this curve with that for the neutral compound it was possible to solve with good agreement the absorption curves

of solutions in which the pH varied from 8.05 to 13.5 and the glycine concentration from 0.06 to 0.5 M.

The diminishing absorption beyond pH 11.1, shown in Fig. 2, *B*, is due in part at least to the precipitation of inorganic cupric hydroxide, since filtration of these solutions left a visible pale blue precipitate on the paper. Kjeldahl analysis of this precipitate showed it to be free of nitrogen. The curves in this pH region are therefore dotted in Fig. 2, *B*.

(b) *Potentiometric Data and Constitution*

First Acid Complex—In Table I are collected the data from E.M.F. measurements, and the calculations of m and r for the first acid copper-glycine; *i.e.*, the number of molecules of glycine combined with 1 of copper, and the number of hydrogen ions set free in the formation of 1 molecule of complex. The data indicate that in this complex 2 molecules of glycine combine with 1 of copper, liberating 1 hydrogen ion.

In the calculation of the instability constant (Column 11) the concentrations of free *Zwitter Ion* glycine (Column 8) and not of total uncombined glycine were taken. This procedure is based on the observations shown in Fig. 2, and discussed in Section I, *c*, that complex formation progressively diminishes with suppression of ionization of the carboxyl group and disappears at high acidity; *i.e.*, only the *Zwitter Ion* glycine participates in this equilibrium.

The absorption spectra of the first two solutions in Table I were analyzed algebraically as described above, and the resulting concentrations of cupric ion and complex are given in Columns 4 and 5. Of the remaining four solutions, in one pair the pH was the same, but the glycine concentrations were different, while in the other the concentrations of glycine were the same, and those of hydrogen ion different. Since it is not possible to decide, *a priori*, the value of r , values of m were calculated for different values of $r = 0, 1$, and 2 , respectively. From Column 10 it is clear that only when r is taken equal to 1 is a concordant value, 2 , obtained for m .

The values for the instability constant K are different for each pair of experiments. This variation is due in the first pair to the uncertainty of the pH values, which were obtained colorimetrically, and in the other solutions is probably due to hydrogen ion

TABLE I

$$\text{Values of } m, r, \text{ and } K \text{ for } \frac{(\text{Cu}^{++}) (\text{Glycine}^{\pm})^m}{(\text{CuGlycine}_m) (\text{H}^+)^r} = K \text{ for First Acid Glycine}$$

 Concentration of CuSO_4 in all solutions = 0.002 M.

Solution No.	pH (2)	Cu electrode E.M.F. (3)	Cu ⁺⁺ (4)		Cu in complex (5)		Total glycine (6)	Ionization of glycine (7)	Free glycine [±] (8)		Values of <i>m</i> (9)			Log $K_{r=1}$ (10)	<i>K</i> (11)
			mol	Log	mol	Log			mol	Log	<i>r</i> = 0	<i>r</i> = 1	<i>r</i> = 2		
G 7	1.8*		0.0014 S	-2.85	0.00058 S	-3.24	0.50	20	0.10	-1.00	3.0	1.8	0.7	+0.15	1.4
G 8	2.0*		0.00084 S	-3.08	0.00116 S	-2.94	0.50	30	0.149	-0.83				+0.20	1.6
G 22	2.24†	+0.0206	0.00020	-3.70	0.00180	-2.74	0.20	44.8	0.088	-1.06	1.9	2.0	2.0	-0.83	0.15
G 23	2.25†	+0.0067	0.00059	-3.23	0.00141	-2.85	0.10	45.4	0.044	-1.36				-0.85	0.14
G 41	1.97†	-0.0048	0.00145	-2.84	0.00055	-3.26	0.40	29.2	0.117	-0.93	4.0	2.5	1.0	+0.51	3.2
G 42	2.27†	-0.0070	0.00058	-3.24	0.00142	-2.85	0.40	46.3	0.185	-0.73	Mean value of <i>m</i> = 2.1			+0.40	2.5

* = pH determined colorimetrically; error ± 0.2 .

† = pH determined by glass electrode; error ± 0.02 .

S = from analysis of absorption spectra.

liquid junction potentials. Since the value of m is based only on the difference in potential between two members of a pair, it is probably unaffected by this junction potential, which will be nearly the same in each member of the pair. The possibility discussed above, of the existence of still another complex in this pH region, may also be responsible for variations in the value of K . The agreement previously found between absorption curves indicates that the range of stability within which this other complex exists, if it exists at all, is very narrow.

The present data, therefore, point to the conclusion that the compound whose absorption spectrum is Curve 3 in Fig. 1, and to which the values of $m = 2$, and $r = 1$ apply, is the principal one found in high acidity.

Second Acid Complex—The conditions under which the second acid copper-glycine exists are too restricted to allow of any reliable determination of the values of m and r by these methods. It appears within only a narrow range of hydrogen ion and glycine concentrations. The minimum variation in glycine concentration necessary for a reliable determination of the value of m causes this compound to disappear in one or other of the solutions. At pH 6, and with glycine concentrations varying from 0.002 to 0.005 M, the absorption spectra indicated that the principal compound was this second acid complex. The value deduced for m from a few measurements made on these solutions was between 2 and 3; and the power of (H^+) was apparently fractional; *i.e.*, 1 H ion was released by the formation of 2 or more complex cupric ions.

The absorption curve of the second acid copper-glycine is nearly identical with that of the second acid copper-alanine. The constitution of the latter compound seems to be firmly established as $Cu_2alanine_3$. From this identity the constitution of the second acid copper-glycine may be taken to be similar; *i.e.*, $Cu_2glycine_3$. Such a constitution would account for the effect of dilution favoring the second acid compound instead of the neutral complex whose formula is $Cu\ glycine_2$, since the compound containing fewer amino acid molecules to 1 of copper would be more stable at lower total amino acid concentrations. This is discussed below in connection with neutral and basic alanine.

Neutral Complex—The data and the calculations of m and r for the neutral copper-glycine are set out in Table II. The data fall

TABLE II
Calculation of m for $\frac{(Cu^{++})(Glycine)^m}{(CuGlycine_m)(H^+)^r} = K$ for Neutral Copper-Glycine

Concentration of $CuSO_4$ in all solutions = 0.002 M.

Solution No.	$CuSO_4$ in E.M.F. stand-ard	Cu elec-trode E.M.F.	Free Cu^{++}		Bound Cu		pH	Total ion-ized gly-cine	Bound glycine for $m = 2$		Free ionized glycine		From solution No.	m	From solution No.	m
			mols	Log	mols	Log					mols	Log				
G 43	0.01	0.056	1.29×10^{-4}	4.11	1.87×10^{-3}	3.272	5.5* ca.	0.025	3.7×10^{-3}		0.021	2.322	G 43 + G 44	2.9	G 45 + G 46	1.9
G 44	0.01	0.0845	1.38×10^{-5}	5.14	1.99×10^{-3}	3.299	5.5* "	0.05	3.98×10^{-3}		0.046	2.663	G 43 + G 45	2.3	G 43 + G 46	2.3
G 45	0.01	0.100	4.17×10^{-6}	5.62	2×10^{-3}	3.301	5.5* "	0.10	4×10^{-3}		0.096	2.982	G 44 + G 45	1.6		
G 46	0.01	0.118	1.05×10^{-6}	6.02	2×10^{-3}	3.301	5.5* "	0.20	4×10^{-3}		0.196	1.292	G 44 + G 46	1.8		
													$r = 1$		$r = 2$	
G 47	0.001	0.130	4.0×10^{-8}	8.60	2×10^{-3}	3.301	6.13†	0.10	4×10^{-3}		0.096	2.982	G 47 + G 48	2.1	G 47 + G 48	1.5
G 48	0.001	0.148	1.0×10^{-8}	8.00	2×10^{-3}	3.301	6.28†	0.16	4×10^{-3}		0.156	1.193				

Mean value of m from Solutions G 43, G 44, G 45, and G 46 ($pH_2 - pH_1 = 0$) = 2.1.

In Solution G 47, $K = 0.25$; in Solution G 48, $K = 0.23$.

* = by electrometric and colorimetric methods described in the text.

† = by glass electrode in solution containing copper.

into two groups. In Solutions G 43 to G 46 the pH was obtained colorimetrically and the cupric ion concentrations given cannot be considered, therefore, as absolute values. However, the differences in potential correspond to actual differences in cupric ion concentration, and the value obtained for m , which is derived only from these differences, we therefore consider reliable. In Solutions G 47 and G 48 the hydrogen ion concentrations were measured by means of the glass electrode in the final solutions containing copper, and the pH of the standard CuSO_4 solution was adjusted to 6.1. The data obtained from these two solutions are therefore the most trustworthy. Both groups of data indicate clearly that the value of m is 2, agreeing with the constitution found for the crystals of neutral copper-glycine in the literature.

On the other hand, these data do not permit of a decision regarding the value of r , since the hydrogen ion concentrations were maintained constant, or varied only slightly, in order to calculate m . When $m = 2$ is substituted in the mass law equation and the data of Solutions G 47 and G 48 are used, a value of 1.1 is obtained for r , but the pH difference is too small for this value to be relied upon. Larger differences in pH are not experimentally feasible here, since it would then be impossible to estimate the true copper electrode potential difference on account of the varying and unknown hydrogen ion liquid junction potentials. This difficulty was circumvented by measuring the change in hydrogen ion concentration after the addition of CuSO_4 to an isoelectric solution of the amino acid containing 0.1 M K_2SO_4 . The increase in hydrogen ion concentration plus the hydrogen ions absorbed by the glycine corresponds to the number of hydrogen ions released in the formation of the copper complex. The ratio of the number of hydrogen ions released to the number of molecules of copper known to be bound gives the value of r . The changes in hydrogen ion concentration were measured by means of the glass electrode.

The procedure was as follows: 0.1 M CuSO_4 was added from a micro burette to 25 cc. of 0.5 M glycine in 0.1 M K_2SO_4 , initially present in the glass electrode vessel. After each addition, with continuous, thorough stirring by means of a current of nitrogen, 3 to 5 minutes were allowed for the attainment of a steady potential which did not drift more than 0.3 millivolt during this interval. These potentials were converted to pH values by means of a

graph constructed from the glass electrode potentials of a series of phthalate buffers extending between pH 3.6 and 6.1. The graph obtained was a straight line so that no uncertainty was incurred in interpolation. Its slope at 23° was found to be 57.6 millivolts per unit difference in pH. At the end of the titration, which as a rule occupied about an hour, the constancy of the glass electrode during the titration was checked by measuring again the potential of one or more of the buffer solutions. The change was never more than 0.3 millivolt.

TABLE III
Number of Equivalents of Hydrogen Ions Set Free in Formation of Neutral Copper-Glycine

25 cc. of 0.5 M glycine containing 0.1 M K₂SO₄ were initially present.

0.1 M CuSO ₄ added	Total Cu in solution ≈ total bound Cu	pH	(H ⁺)	Glycine in undisso- ciated form = bound (H ⁺) 1 - α	Total (H ⁺) liberated (4) + (5)	Ratio (H ⁺) set free Complex formed $\frac{(6)}{(2)}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
cc.	mol per l.		mol per l.	mol per l.	mol per l.	
0	0	6.04				
0.3	0.00119	4.90	0.0000126	0.00131	0.00132	1.1
0.4	0.00158	4.77	0.0000170	0.00177	0.00179	1.1
0.5	0.00196	4.65	0.0000224	0.00231	0.00233	1.2
0.6	0.00234	4.58	0.0000263	0.00271	0.00274	1.2
0.7	0.00272	4.52	0.0000302	0.00308	0.00311	1.1
0.8	0.00310	4.47	0.0000339	0.00345	0.00348	1.1
1.0	0.00385	4.38	0.0000417	0.00418	0.00422	1.1

The total concentration of bound copper in the neutral complex form (Column 2, Table III) was taken as equal to the amount added. The error so incurred is negligible at this pH, when the glycine, as it is here, is greatly in excess of the copper. The undissociated glycine (1 - α) in Column 5 was calculated on the basis of a *Zwitter Ion* pK_a of 2.33, and a value of *m* for the neutral complex of 2. Column 7 shows that 1 hydrogen ion is liberated when 1 cupric ion is bound as neutral copper-glycine. When 1 is used for the value of *r*, the instability constant *K*, from the data of Solutions G 47 and G 48 in Table II, is 0.24.

Basic Complex—We were unable to obtain cupric ion measurements of sufficient reliability at alkalinities at about pH 11 to

attempt determinations of m , r , and K for the basic complex. The following facts can be deduced about it, however.

First, the peak of absorption is still in the orange, though shifted some 400 Å. towards the red (*cf.* Curves 5 and 6, Fig. 1). This may be taken to indicate that whatever inner complex structure is assigned to the neutral complex, that assigned to the basic complex cannot be very different in type, the difference being probably quantitative rather than qualitative. Secondly, the ratio of concentrations of neutral and basic complexes present in a mixture, at any one pH, is not largely influenced by changing the

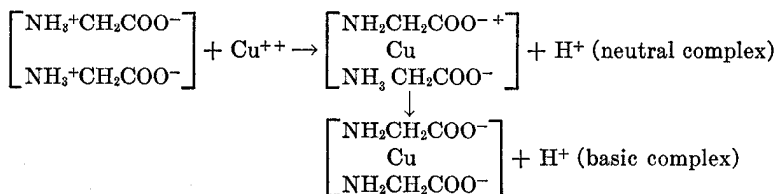
TABLE IV
Dependence of Relative Amounts of Neutral and of Basic Copper-Glycine on Hydrogen Ion Concentration

Solution No.	pH	Total glycine	Per cent total glycine in		Per cent total Cu as	
			$\begin{array}{c} \text{NH}_3^+ \\ \diagup \\ \text{CH}_2 \\ \diagdown \\ \text{COO}^- \\ \text{form} \end{array}$	$\begin{array}{c} \text{NH}_2 \\ \diagup \\ \text{CH}_2 \\ \diagdown \\ \text{COO}^- \\ \text{form} \end{array}$	Basic copper-glycine	Neutral copper-glycine
		<i>mol per l.</i>				
1	8.05	0.5	98	2	94	6
2	8.1	0.05	98	2	99	1
3	8.1	0.002	98	2	100	0
4	8.7	0.5	92	8	85	15
5	8.7	0.05	92	8	90	10
6	9.2	0.1	78	22	79	21
7	9.8	0.5	47	53	41	59
8	10.2	0.5	27	73	29	71
9	11.1	0.5	4	96	12	88

concentration of amino acid (see Solutions 2 to 5, Table IV). This fact indicates that the number of molecules of glycine per molecule of copper is the same in both these complexes. The third point is that, within the limits of accuracy of the pH determinations and absorption measurements involved, the ratio of concentrations of basic and neutral complexes present in a mixture is the same as the ratio of concentrations of basic and neutral forms of glycine.

In Table IV the amounts of the two complexes were obtained by algebraic treatment of the absorption curves, and the proportion of glycine in the basic form is calculated from the pK of 9.75.

It is clear that these proportions agree with the proportions of the basic complex present. In the absence of confirmatory data, therefore, the simplest interpretation would be as follows: the neutral complex is formed from 2 molecules of glycine and 1 cupric ion, with elimination of 1 H ion; the basic complex is formed from this by elimination of a 2nd H ion. That is



This formulation agrees with the finding that the equilibrium between these two complexes is controlled only by the pH and is uninfluenced by the amino acid concentration. The absorption spectrum to be expected would still be of the same general type, though with some change corresponding to the change in charge.

IV. Cupric Salts of Alanine

(a) Absorption Spectra

As with glycine, the absorption curves indicated the existence of four copper-alanine complexes, two in the acid, one in the neutral, and one in the alkaline range of pH. In Fig. 3 the absorptions in the visible spectrum of the four compounds are set out and compared with those of copper sulfate (*i.e.* cupric ion), undissociated copper acetate, and cupriammonium sulfate.

In acid solutions the absorption due to the complex was obtained, as with glycine, by determining the free cupric ion with the copper electrode and subtracting the absorption due to this. The extinction coefficients were then computed, the concentration of complex being that of the total copper minus the free cupric ion. The absorption for the first acid complex was derived from the last four sets of measurements in Table V, the absorption spectra of these solutions being taken at the same time. The curve for the second acid complex was obtained from solutions in which the pH varied from 2.0 to 4.0 and the alanine concentration from 0.005 to 0.5 M.

The absorption curve for the neutral complex is the mean from a large number of solutions in which it was clear that only this complex was present. Twelve solutions, in which the pH varied from 6.5 to 11.1 and the alanine concentration from 0.5 to 0.07 M, gave practically identical absorptions. Fig. 4, which is the alanine analogue of Fig. 2, shows the change in absorption with pH, at constant concentrations of copper and alanine, and also shows the relative constancy of the absorption in the neutral range.

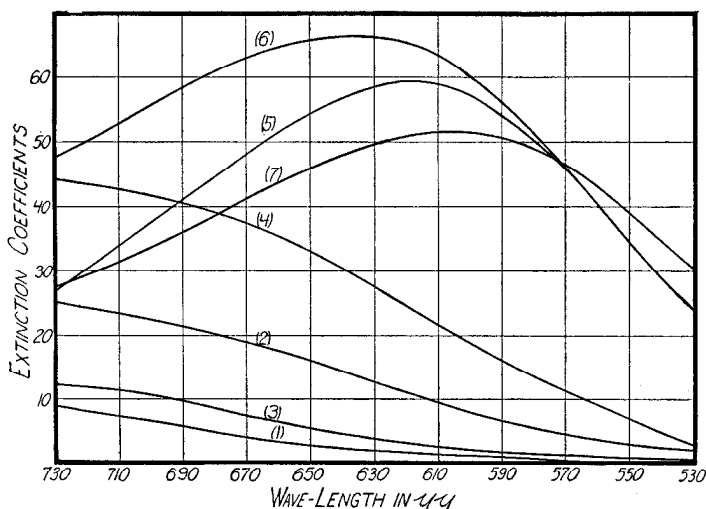


FIG. 3. Absorption curves of cuprialanine compounds. Extinction coefficients, $\log \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{L}$ (cm.) at various wave-lengths in $\mu\mu$. Curve 1, copper sulfate; Curve 2, copper acetate in alcohol; Curve 3, first acid cuprialanine; Curve 4, second acid cuprialanine; Curve 5, neutral cuprialanine; Curve 6, basic cuprialanine; Curve 7, cupriammonium sulfate.

Curve 6 in Fig. 3 is the mean of the twelve curves thus obtained. With this curve and those of the first and second acid complexes, together with the curve for CuSO_4 , it was possible to account for the absorptions of all the other solutions in the acid range up to pH 6.8, and with alanine concentration from 0.002 to 0.5 M, the copper being always constant at 0.002 M.

In alkaline solutions the change in absorption with pH was different from that found with glycine. As Fig. 4, B shows, with

alanine 0.1 M very little increase in absorption occurs even up to pH 11; *i.e.*, the basic compound either has an absorption curve similar to that of the neutral compound or else is not being formed at this dilution. The latter alternative is the true explanation, for at pH 11.1 the absorption in the red steadily increased as the

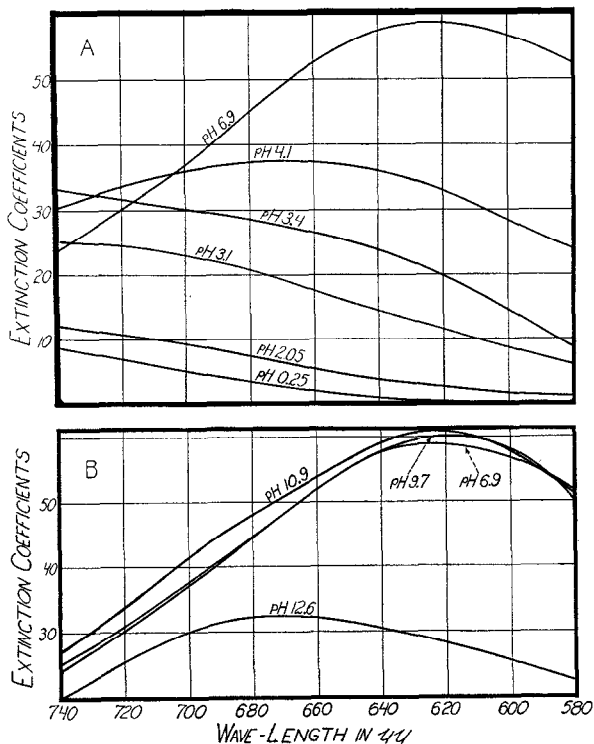


FIG. 4, A. Effect of changing H ion concentration on the absorption of cuprialanine solutions containing the same amounts of copper and of alanine; Cu = 0.002 M, alanine = 0.10 M; acid range.

FIG. 4, B. Same as Fig. 4, A; alkaline range.

alanine concentration was increased. Fig. 5 shows this change from a 7 times excess of alanine over copper concentration—which gives a curve almost identical with that of the neutral complex—to a 500 times excess. The absorption of the solution containing 500 times excess alanine over copper was the same as that of a solution containing 0.001 M copper and 0.9 M alanine,

i.e. a 900 times excess, at the same pH. This curve was therefore considered to be that of the pure basic complex. Its accuracy was corroborated by the concordant results obtained when it was employed, with the absorption curve of the neutral copper-alanine, in the analysis of the absorption of all other solutions in the alkaline range. In extreme alkalinity, as with glycine, precipitation occurred, and the absorption was correspondingly lowered.

Comparison of Figs. 4, *A* and 4, *B* with Figs. 2, *A* and 2, *B* shows that the change of absorption with pH in the acid ranges is very similar with alanine and glycine, while in the alkaline range

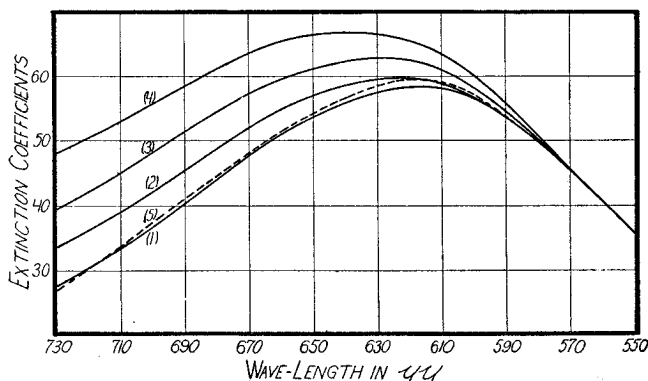


FIG. 5. Effect of increasing alanine concentration at constant pH = 11.1, Cu = 0.002 M. Curve 1, alanine 0.016 M; Curve 2, alanine 0.20 M; Curve 3, alanine 0.50 M; Curve 4, alanine 1.0 M; Curve 5, neutral cupri-alanine (*cf.* Fig. 3).

a considerable difference is noticeable. The significance of this is further discussed below.

(b) Potentiometric Data and Constitution

First Acid Copper-Alanine—The values of m and r for this complex were obtained from the data in Table V. A concordant and reasonable value for m was obtained only when r was taken equal to 0; m is then clearly 2. This complex therefore differs from the first acid copper-glycine, in which 1 hydrogen ion is released. A corresponding difference appears in the absorption curves of these two complexes. The curve of cupric alanine, for

TABLE V
Calculation of m , r , and K for $\frac{(Cu^{++})(Alanine)^m}{(CuAlanine_m)(H^+)^r} = K$ for First Acid Copper-Alanine

Solution No.	Total $CuSO_4$	Cu^{++} in stand-ard solution	Cu electrode E.M.F.	Free Cu^{++}		Bound Cu		pH	Total alanine	Total alanine ionized	Bound alanine for $m = 2$		Free ionized alanine		From solution No.	m $r = 0$	$K \times 10^4$
				Log	mol	Log	mol				mol	mol	Log	mol			
A 20	0.002	0.001	0.00066 S	4.82	0.00134	3.127	2.05*	0.1	0.034	0.003	0.031	2.491			A 20 + A 21	2.1	4.7
A 21	0.002	0.001	0.0005 S	4.699	0.0015	3.176	2.5*	0.1	0.040	0.003	0.037	2.568					4.6
A 34	0.002	0.001	0.00126	3.10	0.00074	4.869	1.40†	0.25	0.028	0.001	0.027	2.431			A 34 + A 33	1.8	12
A 33	0.002	0.001	0.00046	4.66	0.00154	3.188	1.97†	0.25	0.075	0.003	0.072	2.857			A 33 + A 31	2.2	15
A 31	0.002	0.001	0.00096	5.98	0.00191	3.281	1.99†	0.5	0.16	0.004	0.16	1.204			A 34 + A 31	2.0	14
A 32	0.002	0.001	0.00033	4.52	0.00167	3.22	1.51†	0.5	0.065	0.003	0.062	2.792			A 34 + A 32	2.2	8

S = from analysis of spectrophotometric data only.

* = determined by means of hydrogen electrode and colorimetrically as described in text.

† = determined by means of glass electrode.

TABLE VI
Calculation of a , m , r , and K for $\frac{(Cu^{++})^a(Alanine)^m}{(Cu_aAlanine_m)(H^+)^r} = K$ for Second Acid Copper-Alanine

Solution No.	Total $CuSO_4$	Cu^{++} in stand-ard solution	Cu electrode E.M.F.	Free Cu^{++}		Bound Cu		pH	Total alanine	Total alanine ionized	Bound alanine $a = 2$ $m = 3$		Free ionized alanine		From solution No.	m $r = 1$ $a = 2$	$K \times 10^4$
				Log	mol	Log	mol				mol	mol	Log	mol			
A 103	0.002	0.001	-0.0070	0.00172	3.235	0.00028	4.447	4.00†	0.0025	0.00245	0.00042	0.00203	3.308		A 103 + A 104	2.7	9
A 104	0.002	0.001	-0.0051	0.00148	3.170	0.00052	4.716	3.88†	0.004	0.00388	0.00078	0.00310	3.491		A 104 + A 106	3.3	9.5
A 106	0.002	0.001	-0.0036	0.00076	4.88	0.00124	3.093	3.92†	0.008	0.00776	0.00186	0.00590	3.771		A 103 + A 106	3.1	9

† = pH determined by means of glass electrode on final solution containing copper.

which $r = 0$, is very low in the visible spectrum, resembling the curve of CuSO_4 , while that for the copper-glycine, for which $r = 1$, is much higher. The former approximates the curve of a simple salt; the latter that of a "complex."

The values of K derived from spectrophotometric data (Solutions A 20 and A 21, Table V) are distinctly lower than those derived from potentiometric measurements. Probably, as in the case of glycine, the discrepancy is due to the hydrogen liquid junction potentials. However, the variation in the value of K , in view of the experimental difficulties in these solutions, is not great, and its mean value may be taken to be close to 1×10^{-3} .

TABLE VII

Calculation of Number of Hydrogen Ions Released in Formation of Second Acid Copper-Alanine

Ala- nine	pH after addi- tion of 0.002 M CuSO_4	(H^+)	$\frac{1-\alpha}{\alpha} =$ $\text{pK} - \text{pH}$	Fraction of alanine undis- sociated $1-\alpha$	(H^+) bound by alanine	Total (H^+) released (3) + (6)	Total (Cu) bound	Ratio (H^+) set free Cu in complex (7) (8) (9)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
<i>mol</i> <i>per l.</i>		<i>mol per l.</i>			<i>mol per l.</i>	<i>mol per l.</i>	<i>mol per l.</i>	
0.0020	3.98	0.000105	2.35	0.022	0.000044	0.000149	0.00038	0.4
0.0025	3.99	0.000099	2.34	0.022	0.000055	0.000154	0.00028	0.6
0.0040	3.99	0.000102	2.34	0.022	0.000088	0.000190	0.00049	0.4
0.0040	3.90	0.00013	2.43	0.026	0.000104	0.000234	0.00052	0.5
0.0050	3.94	0.00012	2.39	0.024	0.00012	0.00024	0.00054	0.5
0.0080	3.80	0.00016	2.53	0.033	0.000261	0.000421	0.00124	0.4
0.0100	3.84	0.00014	2.49	0.030	0.00030	0.00044	0.00085	0.5

Second Acid Complex—The second acid alanine complex presented the same difficulty as the corresponding glycine compound; *i.e.*, the restricted range of concentrations of amino acid and of hydrogen ion in which this form exists free of other complexes. After a number of trials a few solutions containing only this compound in equilibrium with cupric ions were obtained. The resulting data are given in Table VI. Concordant values for m , r , and K were obtained only when the reaction was taken to be



(*i.e.*, m has the value 1.5, or is 3 if $a = 2$, where a is the number of Cu atoms in the complex. Also, if $a = 2$, $r = 1$).

Further corroboration of this formula is found in the results of direct estimation of the hydrogen ions set free when this complex is formed (Table VII). The hydrogen ion activities in these solutions were measured by means of the glass electrode, free cupric ion concentrations by means of copper electrode potentials. Spectrophotometric measurements showed that the major part of the bound copper was in the form of the second acid complex. The ratio of the numbers of hydrogen ions set free to cupric ions bound (Column 9) is clearly 0.5; *i.e.*, if $a = 2$, $r = 1$.

The absorption curve of the second acid copper-alanine is nearly identical with that found by trial and error for the corresponding copper-glycine. This coincidence suggests, as pointed out above, that the constitution of the latter compound is similarly $\text{Cu}_2\text{glycine}_3$. The findings, mentioned above, that for the second acid glycine complex, r , the power of (H^+) , appeared to be fractional and that m appeared to be of the order of 2 or 3, also support this formula. In the absence of other evidence, we have therefore assumed it to be correct.

Neutral Copper-Alanine—The electrometric data for the neutral complex (Table VIII) fall into two groups: those derived from solutions at pH about 6.8, and those at pH 7.9. The hydrogen ion liquid junction potentials are again probably responsible for the discrepancy in the values of K . The value of m is either 2.5 or 3; *i.e.*, the complex has the constitution Cu alanine_3 or $\text{Cu}_2\text{ alanine}_5$. In either case, the direct estimation of the hydrogen ions set free in the formation of this compound, given in Table IX, shows unequivocally that 1 hydrogen ion is set free for each cupric ion bound. In this respect the complex resembles the corresponding copper-glycine. The power of (H^+) was accordingly taken as 1 in the calculation of m in Table VIII. The absorption curve of this complex is somewhat higher than that of the neutral copper-glycine, though the maximum is in nearly the same part of the spectrum. A possible explanation for these facts is that the higher absorption of neutral copper-alanine is due to the larger number of molecules of amino-acid relative to copper in its composition, but that the number of nitrogen atoms attached to copper, which according to Kober and Haw governs the position of the peak of absorption, is the same in the two compounds.

Basic Copper-Alanine—The basic alanine complex is much

TABLE VIII
 $(Cu^{++})^a (Alanine)^m$
Calculation of a, m, and r for $(Cu_a, Alanine_m) (H^+)^r = K$ for Neutral Copper-Alanine

Solution No.	Cu ⁺⁺ in standard solution	Cu electrode E.M.F.	Free Cu ⁺⁺		Bound Cu		pH	Total alanine (all ionized)	Bound alanine $m = 3$	Free alanine (all ionized)		From solution No.	m $r = 1$	Log K	
			$mols$	Log	Log	$mols$				Log	Log				
A 114	10^{-4}	+0.100	4.17×10^{-8}	8.62	0.002	3.301	6.71†	0.4	0.006	0.394	I.596	A 114 + A 115	3.3	0.8	1.0
A 115	10^{-3} 10^{-4}	+0.0835 +0.0728	3.47×10^{-7}	7.54	0.002	3.301	6.81†	0.2	0.006	0.194	I.288	A 114 + A 116	3.0	0.9	1.3
A 116	10^{-4}	+0.0427	2.0×10^{-6}	7.30	0.002	3.301	6.89†	0.1	0.006	0.094	2.973	A 115 + A 116	2.6	0.8	1.3
A 117	10^{-5}	+0.156	5.37×10^{-11}	11.73	0.002	3.301	7.91†	0.25	0.006	0.244	I.387	A 117 + A 118	2.5	-1.5	-1.2
A 118	10^{-5}	+0.110	4.47×10^{-9}	9.28	0.002	3.301	7.93†	0.0625	0.006	0.0365	2.752	A 117 + A 119	2.5	-1.8	-1.2
A 119	10^{-5}	+0.050	2.04×10^{-7}	7.31	0.002	3.301	7.89†	0.0156	0.006	0.0096	3.982	A 118 + A 119	2.6	-2.1	-1.1

† = pH measurements made with glass electrode in final mixtures containing copper.

more sensitive to changes in the concentration of amino acid than is the basic glycine compound. At pH 11.1, increase in the concentration of alanine from 0.2 to 1.0 M changed the amount of the basic complex (by analysis of the curves in Fig. 5) from 25 to 100 per cent of the total copper. This indicates that the number of

TABLE IX
Number of Equivalents of Hydrogen Ions Set Free on Formation of Neutral Copper-Alanine

Experiment I. 25 cc. of 0.4 M alanine containing 0.1 M K₂SO₄.
" II. 25 " " 0.5 " " " 0.1 " "

Experi- ment No.	CuSO ₄ added	Cu in complex formed	pH	(H ⁺)	Fraction of alanine undis- sociated $1 - \alpha$	Alanine undis- sociated	Total (H ⁺) set free (5) + (7)	Ratio (H ⁺) set free Complex formed $\frac{(8)}{(3)}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	cc. 0.1 M	mol per l.		mol per l.		mol per l.	mol per l.	
I	0	0	6.08					
	0.20	0.00079	5.02	0.0000096	0.00204	0.000813	0.000823	1.04
	0.30	0.00118	4.85	0.0000141	0.00307	0.00120	0.00121	1.03
	0.40	0.00157	4.72	0.0000191	0.00407	0.00162	0.00164	1.04
	0.50	0.00196	4.62	0.0000240	0.00510	0.00203	0.00205	1.04
	0.60	0.00234	4.54	0.0000289	0.00613	0.00242	0.00245	1.05
	0.70	0.00272	4.46	0.0000347	0.00735	0.00290	0.00293	1.07
	0.80	0.00310	4.41	0.0000389	0.00826	0.00324	0.00328	1.06
	1.00	0.00384	4.33	0.0000468	0.01000	0.00385	0.00390	1.02
II	0.30	0.00118	5.07	0.0000085	0.00182	0.00091	0.00092	0.78
	0.40	0.00157	4.88	0.0000130	0.00281	0.00138	0.00139	0.89
	0.50	0.00196	4.76	0.0000174	0.00370	0.00181	0.00183	0.93
	0.60	0.00234	4.67	0.0000214	0.00455	0.00221	0.00223	0.95
	0.70	0.00272	4.60	0.0000251	0.00534	0.00260	0.00263	0.97
	0.80	0.00310	4.55	0.0000282	0.00599	0.00292	0.00295	0.95
								Mean 0.99

molecules of alanine bound to 1 of copper is not the same in the neutral and basic compounds. From the expressions

$$\frac{(\text{Cu}) (\text{alanine})^x}{(\text{Cu alanine}_x) (\text{H}^+)^{r'}} = K_n \text{ for the neutral complex}$$

and

$$\frac{(\text{Cu}) (\text{alanine})^y}{(\text{Cu alanine}_y) (\text{H}^+)^{r''}} = K_b \text{ for the basic complex}$$

it follows that at any one pH, where both complexes are present, the term for Cu ion vanishes, and the expression

$$\frac{(\text{Cu alanine}_y)}{(\text{Cu alanine}_x)} = k \cdot (\text{alanine})^{y-x} \cdot (\text{H}^+)^{r'-r''}$$

is obtained, where $k = \frac{K_n}{K_b}$. Hence at constant pH, if y is greater than x , the proportion of basic to neutral complex will become greater with increasing alanine concentration. This is, in fact, what occurs, so that the value of m for the basic copper-alanine is therefore greater than 2.5 or 3, according to which figure is taken for the neutral complex. In the case of glycine the distribution of copper between neutral and basic complexes was shown above to be almost independent of amino acid concentration, and hence it was concluded, by similar reasoning, that the number of molecules of glycine bound by 1 of copper is the same in the neutral as in the basic complex.

V. DISCUSSION

(a) *Constitution of Complexes*

The properties of the eight complexes described here are summarized in Table X, with their ranges of stability, empirical formulæ, and references to the data. We have not set down definite structural formulæ because we feel that the data so far obtained are insufficient. Nevertheless, certain deductions may be made regarding their structure from the characteristic absorption spectra of the four types of compounds formed, on the basis (a) of the rule proposed by Kober and Haw, and (b) of a second rule suggested by our observations, that the height of an absorption curve, without reference to its position in the spectrum, is greater the larger the number of amino acid molecules in the complex.

The spectra of the first acid complexes show a low absorption in the visible portion limited to the red and orange (Figs. 1 and 3). By application of the above rules, the copper in these compounds therefore is linked only to the oxygen atoms of the carboxyl groups; *i.e.*, they approximate undissociated salts. The peaks of their curves are presumably far in the infra-red. The maximum absorption of the second acid complexes, on the assumption that

TABLE X
Summary of Copper Complexes of Glycine and Alanine

Complex (1)	Absorption (2)	Formula (3)	Range of stability (4)	Probable formula of complex (5)	H ions set free per atom Cu bound (6)	Absorption characteristics (7)
1st acid glycine.....	Fig. 1	Table I	<i>Ca.</i> pH 0.5-2.5	Cu glycine ₂	1	Curve similar to Cu- (OAc) ₂ in alcohol
2nd "	" 1		pH 2-5; in high dilu- tion, up to pH 7	Cu ₂ glycine ₃	0.5	Peak just in infra-red
Neutral glycine.....	" 1	Tables II, III	pH 5-8; overlapping basic complex to pH 10.5	Cu glycine ₂	1	" at 6250 Å.
Basic "	" 1	Table IV	pH 8-12; not affected by dilution	Cu glycine ₂	2	" " 6700 "
1st acid alanine	" 3	" V	<i>Ca.</i> pH 0.5-2.5	Cu alanine ₂	0	Very low absorption; about half that of 1st acid glycine and close to that of cu- pric ion
2nd "	" 3	Tables VI, VII	pH 2.5-6; favored by dilution	Cu ₂ alanine ₃	0.5	Exactly similar to 2nd acid glycine
Neutral "	" 3	Tables VIII, IX	pH 5-9; in dilute solu- tions to pH 11	Cu alanine ₂ or Cu ₂ alanine ₃	1	Peak at 6200 Å.; higher than neutral glycine
Basic "	Figs. 3, 5		pH 8-11; only in con- centrated solutions	Cu alanine ₂	2	Peak at 6460 Å.; higher than basic glycine

the form of all the curves is the same, may be taken to be in the very near infra-red. This suggests the first appearance of a Cu—N bond, which the liberation of 1 hydrogen for every 2 cupric ions bound also indicates. In the case of both neutral complexes 1 hydrogen ion is released for every cupric ion bound. The number of Cu—N bonds of this type is therefore twice as great as in the second acid compounds, which is in accord with the observed shift of the absorption maximum to approximately 6200 Å.

As indicated in Column 5, Table X, an unexpected difference occurs between glycine and alanine in the number of amino acid molecules bound per atom of copper in the neutral and probably also in the basic complexes. Tentatively we have interpreted the higher absorption of the alanine compounds to be due to the larger number of amino acid molecules in the complex.

(b) Application to Donnan Equilibria

It is probable that the variety of complexes formed by protein with bi- and polyvalent cations is at least as great as that found between copper and glycine and alanine. This phenomenon complicates the calculation of Donnan equilibrium relations with proteins. Since the type and extent of complex formation as in the case of amino acids probably varies throughout the whole pH range usually studied, a continually varying correction is necessary for the ions bound in undissociated complexes to the protein. This must be one of the factors responsible for the divergence of the values for the ratios of the calcium concentrations on two sides of a semipermeable membrane from those calculated by means of the unmodified Donnan equation when protein is present on one side (20). Complex formation probably accounts also for the findings of Pincus and Kramer (21) who showed that though the distribution of the monovalent ions between cerebrospinal fluid and serum was in accord with the Donnan equilibrium, the calcium distribution was markedly different.

There are indications that this complication of simple Donnan equilibrium relations by complex formation occurs even with monovalent ions (22). Since complex formation occurs least in highly acid solutions where ionization of the acid groups is suppressed, it would be expected that in this zone simple Donnan equilibrium relations would hold best. This has, in fact, been observed by

Bonino and Garello (23) in their study of Donnan equilibria between ovalbumin and cobaltous salts. At low pH values, the Donnan relation was found to be obeyed; as neutrality was approached, the divergence of the ion concentrations from the calculated values became progressively greater.

SUMMARY

1. The equilibrium relations existing in solution at room temperature between cupric ions and glycine and alanine have been studied by measurement of the absorption in the visible spectrum, and by copper electrode potentials, through a range of hydrogen ion concentrations from pH 0 to 13.

2. The variations in absorption indicate the existence of at least four types of complexes with both glycine and alanine. According to the range of hydrogen ion concentration in which each predominates they have been designated as first acid, second acid, neutral, and basic complexes. The approximate ranges of hydrogen ion and relative amino acid concentrations, in which each type of complex predominates, are delineated.

3. The absolute absorption spectra, in the visible region, of each of the eight complexes in a pure state have been determined. In some cases these were obtained directly, in others by deduction from mixtures. From these curves the absorption spectra of all the mixed systems examined could be derived.

4. The constitution of these eight compounds has been deduced from potentiometric and spectrophotometric data. Of these the constitutions of the second acid glycine, neutral alanine, and basic alanine are uncertain.

5. The number of hydrogen ions set free in the formation of all except the two basic compounds was estimated. The orders of magnitude of the instability constants of the first acid and neutral copper-glycines and of the first acid, second acid, and neutral copper-alanines were determined.

6. The significance is discussed of the phenomena described here in Donnan equilibria involving proteins.

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